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(57) Abstract

Monoclonal antibody Helix–1, produced by hybridoma 2H11, and immunoreactive with an antigen of Helicobacter pylori with an apparent molecular weight of 16 +/– 2kD. An antigenic preparation of a molecule with a molecular weight of 16 +/– 2kD, obtained from Helicobacter pylori. Methods for the qualitative and quantitative detection of said antigen, e.g. by using said antibody.

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HELICOBACTER PYLORI ANTIGEN HAVING AN APPARENT MOLECULAR WEIGHT OF 16±2 KDA, A SPECIFIC ANTIBODY, AND ITS USE FOR THE DETECTION OF SAID ANTIGEN

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TECHNICAL FIELD

The present invention generally relates to:

a) a hybridoma that produces a monoclonal antibody that immunoreacts with an antigen of *Helicobacter pylori* having an apparent molecular weight of 16 ± 2kDa;

b) the monoclonal antibodies produced by said hybridoma;

c) diagnostic methods and systems employing monoclonal antibodies or polyclonal antibodies reacting with a 16 ± 2kDa *Helicobacter pylori* antigen;

d) an antigenic preparation of a molecule with an apparent molecular weight of 16 ± 2kDa obtained from *Helicobacter pylori* and methods to use this antigenic preparation in diagnostic assays relating to *Helicobacter pylori* infections;

e) the use of the specific immunological recognization of a monoclonal antibody, named Helix-1 and produced by a hybridoma named 2H11, or of monoclonal and/or polyclonal antibodies directed against the 16 ± 2kDa *Helicobacter pylori* antigen to detect, in a solid or liquid sample, the presence of *Helicobacter pylori* and/or of the 16 ± 2kDa *Helicobacter pylori* antigen and/or 16 ± 2kDa *Helicobacter pylori* antigen aggregates and/or 16 ± 2kDa *Helicobacter pylori* antigen fragments;

f) the qualitative and/or quantitative determination of a substance produced by *Helicobacter pylori* having an

apparent molecular weight of 16 ± 2 kDa when determined by electrophoresis on the polyacrylammide gel in presence of sodiumdodecylsulfate. Said substance is very specific to *Helicobacter pylori* and then it represents a marker of the presence of the bacterium in the analysed sample or in the environment from which the sample comes;

g) analytical methods to detect the presence of a substance of *Helicobacter pylori* having an apparent molecular weight of 16 ± 2 kDa, in a solid or liquid sample, coming from an organism or the environment or from a culture in vitro.

BACKGROUND ART

Helicobacter pylori is a curve Gram-negative bacterium which is considered to be the most important cause of gastritis and peptic ulcer disease in humans.

There is also evidence that *Helicobacter pylori* infection is associated with gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma.

It is also believed that said infection is associated with the rising of a plurality of cardiocirculatory pathologies, such as the myocardic infarct.

The infection caused by this organism is very frequent and usually persists for life unless treated with antimicrobial drugs.

Eradication of the infection results in the recovery of peptic ulcer and it prevents relapses; consequentely it is now considered to be the first-line therapy for persons suffering from duodenal ulcer.

At present, the most effective cures used to eradicate the infection are based on drugs combinations.

The most important problem of present therapies consists

in the resistance to the used compounds, in particular to metronidazole.

A further very important aspect which is object of careful studies relates to the modalities of infection transmission.

It is believed that they are of interpersonal type (oral-oral or oral-fecal) or derived from external carriers or sources (e.g. foods, contacts with animals).

Helicobacter pylori gastric infection can be detected by looking for the bacterium directly on the bioptical sample drawn during gastro-duodenumscopy.

In this case the methods which can be used are the culture of the organism, the search of the bacteric urease, the simple histologic test.

The culture is considered to be the most specific test since the bacterium identification is based on morphological (curve and spiral shape, Gram-negativity) and biochemical (positivity to catalysis, oxidase, urease and alkalinic phosphatase; negativity to ippurate hydrolysis and to nitrate reduction) criteria.

The most important problem relating to cultural tests is the contamination caused by other bacteria or fungi, problem which can be partially solved by using substrates as more selective as possible by means of antibiotics additions.

Furthermore, tests are available which can detect the *Helicobacter pylori* infection without looking for it in bioptical drawings; they relates to the antibodies search in the serum and the "urea breath test" (search of marked CO_2 in the breath).

The latter are named "non-invasive methods" since they do not need gastroscopy.

Each of invasive and non-invasive methods cited above has advantages and disadvantages, in terms of sensitivity,

specificity, practicalness and costs.

The *Helicobacter pylori* detection is even more difficult when it is to be detected outside of the stomach, e.g. inside of feces or of the oral cavity, where the microbic flora is very rich and heterogeneous and then isolation is very difficult to obtain.

Similar difficulties are present when the bacterium has to be isolated from possible infection sources or carriers (water, food, ground, etc.).

Thus alternative methods for detecting the bacterium are necessary which can substitute or integrate the previous methods cited above with regard to diagnostic and epidemiologic aspects.

Other options are constituted by using immunodiagnostic assay.

The antigen-antibody reaction is the basis for all immunological test methods.

Antibodies are produced by an animal in response to the presence of a substance foreign to the organism (antigen).

The immunological response to an antigen has led to the development of a certain number of techniques which are used to diagnose human and animal diseases.

In order to detect the presence of microrganisms (bacteria or viruses) in body fluids and tissues (urine, serum, plasma, tissue samples or the like), liquids or solids (foods, selective substrates, ground, etc) immunological test methods can be used.

In vitro tests to detect the presence of a predetermined antigen or antibody in a sample are carried out by adding the immunological counterpart to a solution containing the sample to be assayed, i.e. adding antibodies if it is desired to detect the presence of the antigen or viceversa.

Document US-A-4,882,271 discloses a preparation of *Campylobacter pylori* antigen and the use thereof in serum tests.

In this document an antigenic preparation is claimed which is constituted by molecules with molecular weights ranging from 300 to 700 kDa and with isoelectric points ranging from 5,9 to 6,3.

In document PCT/IE95/00037 an antigenic preparation of *Helicobacter pylori* is disclosed, depleted of all the antigens having molecular weight lower than 30kDa, as well as its use in serum tests to search for antibodies directed against *Helicobacter pylori* in persons affected by *Helicobacter pylori* infection.

In document US-A-5,262,156 a further antigenic preparation of *Helicobacter pylori* is disclosed, obtained by means of a plurality of purification phases and comprising molecules with molecular weight of 116, 84, 19 and 14kDa.

This antigenic preparation is used to detect antibodies anti-*Helicobacter pylori* in persons affected by *Helicobacter pylori* infection.

Document PCT/US93/01558 discloses a vacuolating toxin, with molecular weight higher than 972kDa, purified by *Helicobacter pylori*, and its use as a vaccine.

Moreover methods for serum tests and for antigen searches are described.

Document PCT/US92/03284 discloses useful materials and methods for the diagnosis and the therapy of gastric diseases caused by *Helicobacter pylori*.

More particularly a method is claimed for gauging a person's IgE that react with bacteric allergens comprising those of *Helicobacter pylori*.

Document PCT/US94/14239 claims a preparation of antigens

useful for the diagnosis of *Helicobacter pylori* infection by means of serum test.

More particularly methods are claimed to detect a reaction occuring between a person's immunoglobulins and the antigens isolated from a library of antigens specific of the micro-organism with particular care to *Helicobacter pylori*.

In document PCT/SE94/00021 an antigenic preparation of *Helicobacter pylori* is disclosed for the therapy and prophylaxis of *Helicobacter pylori* infection.

In particular the antigenic preparation includes a protein with molecular weight of 120kDa and fragments od said protein with molecular weights of about 20kDa.

Moreover vaccines, monoclonal and polyclonal antibodies against said preparation and methods to detect *Helicobacter pylori* are claimed.

All these documents disclose both the preparation of antigen mixtures of *Helicobacter pylori* and the use of said mixtures in serum tests to detect the antibodies directed against *Helicobacter pylori* in persons affected by said infection.

However, none of said documents teaches the following aspects:

a) the use for a diagnostic purpose of an antigen with apparent molecular weight of 16 ± 2kDa of *Helicobacter pylori* in order to the search for the bacterium;

b) a hybridoma producing monoclonal antibodies directed against an antigen with apparent molecular weight of 16 ± 2kDa of *Helicobacter pylori*;

c) the search, by means of chemical-physical or immunological tests, of the antigen with apparent molecular weight of 16 ± 2kDa of *Helicobacter pylori* in order to diagnose the

presence of *Helicobacter pylori* in the sample to be assayed or in the place from which the sample comes.

DESCRIPTION OF THE INVENTION

According to a first aspect, the present invention relates to an antigen of *Helicobacter pylori* with apparent molecular weight of 16 ± 2kDa and to its use for the search of the *Helicobacter pylori*.

According to another aspect, the present invention relates to a monoclonal antibody which specifically reacts with an antigen of *Helicobacter pylori* with apparent molecular weight of 16 ± 2kDa.

The invention is based on the principle that the presence of this particular *Helicobacter pylori* antigen in any kind of sample indicates the bacterium presence in the place from which the bacterium comes.

This place can be a human or animal organism, or the environment, e.g. food, or a culture in vitro for diagnostic scope.

The antigen can be searched for with immunological and/or chemical-physical techniques.

Another aspect of this invention concerns a hybridoma named 2H11; this hybridoma 2H11 is object of deposit ECACC (Salisbury) n. 96122033 in date 02/12/1996.

The monoclonal antibody obtained by the hybridoma 2H11 is indicated as Helix-1.

According to another aspect, this invention concerns monoclonal antibodies, indicated as Helix-1 and produced by the hybridoma 2H11, that specifically immunoreact with an antigen of *Helicobacter pylori*, which has an apparent molecular weight of 16 ± 2kDa, and which are secreted by the hybridoma 2H11.

Furthermore the presents relates to analytical methods for detecting the presence of the *Helicobacter pylori* antigen with an apparent molecular weight of 16 ± 2kDa.

The antigen is specifically and univocally identified by the monoclonal antibody Helix-1.

Moreover, the invention concerns the use of the specific immunological recognization of the monoclonal antibody Helix-1, or of fragments thereof or of monoclonal and/or polyclonal antibodies or fragments thereof capable of specifically recognizing the antigen having apparent molecular weight of 16 ± 2kDa to detect in a sample the presence of *Helicobacter pylori*, or of the antigen of 16 ± 2kDa or of aggregates of antigen of 16 ± 2kDa, or of fragments of antigen of 16 ± 2kDa.

These and other aspects of the invention are achieved by providing a monoclonal antibody Helix-1 that binds by means of an antigen/antibody reaction to an antigen with the apparent molecular weight of 16 ± 2kDa obtained by the *Helicobacter pylori*.

According to the invention, the antibody Helix-1 shows a high specificity towards the *Helicobacter pylori* since it does not recognize antigens produced by the plurality of assayed bacteric and fungi species: *Campylobacter, Pseudomonas, Escherichia coli, Proteus, Salmonella, Enterococco, Stafilococco, Candida, Neisseria, Klebsiella e Streptococco.*

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Description of the method for producing hybridoma 2H11 and of the characterization of monoclonal antibody Helix-1 secreted by hybridoma 2H11

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Production of hybridoma 2H11

a) Immunization and fusion

Female Balb/c mice from Charles River were immunized with a sonicate solution of *Helicobacter pylori* (about 10^8 cells per milliliter), given subcutaneally in a volume of 500 µl (day 0).

Two boost immunization were administered on day 14 and 28 with the same amount of antigen.

Three days before cell fusion animals were finally injected with 500 µl of *Helicobacter pylori* suspension intraperitoneally.

Fusion of spleen cells with myeloma cell line X63-Ag8.653 was carried out according to Kohler G. and Milstein C. (Nature 256:495-497, 1975).

Growth and selection of fused cells in tissue culture plates were carried out according to standard methods (Goding, 1983).

Supernatants were taken and screened for anti-HP antibody production from all wells exhibiting cell growth, in order to single out the hybridomas producing specific antibodies.

To this aim the technique described in the following paragraph b) has been used.

b) Screening of hybridomas producing anti-HP monoclonal antibodies.

An antigenic preparation constituted by a sonicated *Helicobacter pylori* suspension was diluted 1:1000 (v/v) with carbonate buffer 50 mM, pH 9,6 and 100 microlitres of the dilution were incubated for 16 hours in the wells of a microtiter plate (Maxisorp, Nunc).

After washing the wells of the microtiter plate by means of a physiological solution, 200 microlitres PBS (pH 7,4) containing 10 mg/ml bovine albumine (BSA, fraction V, Sigma) were added to the wells.

The plate was incubated again for 16 hours at 4°C.

After washing by means of physiological solution, 100 microlitres of the cellular supernatants were added to the wells of the previously prepared microtiter plate, and they were incubated for 2 hours at room temperature.

The wells were carefully washed with PBS, and 100 microlitres of horseradish peroxidase conjugated goat anti-mouse immunoglobulins-HRP labeled were added to each well.

After one hour incubation at room temperature the wells were washed with PBS.

200 microlitres sodium citrate buffer 0,1M, pH 4,75 containing 1 mg/ml urea peroxide and 1 mg/ml ophenylendiamine HCl (OPD) were added to the wells.

After 15 minutes the enzymatic reaction was stopped by means of 50 microlitres 2N Hcl, and the absorbances were gauged at 492 nm.

c) Preparation of Helix-1 anti-HP monoclonal antibodies.

The ascites fluids containing the monoclonal antibodies according to the present invention was obtained by intraperitoneally inoculating 10^6 cells of hybridoma 2H11 into 10 weeks old Balb/c mice, that had previously been primed with 0,5 ml pristane.

The average developing time of the ascites was 15 days.

The purified monoclonal antibody Helix-1 was prepared by means of affinity cromatography on a Protein A-Sepharose CL-4B column (Pharmacia Biotech, Uppsala, Sweden).

d) Isotyping of monoclonal antibody Helix-1

The supernatant derived by cloned cells of hybridoma 2H11 was used for isotyping monoclonal antibodies Helix-1 by using a mouse hybridoma isotyping kit manufactured by Bio-Rad Laboratories (Milan, Italy).

Antibody Helix-1 produced by hybridoma 2H11 belonged to immunoglobulinic isotype IgG1.

e) Characterization of the molecular weight of the antigen immunologically bound by monoclonal antibody Helix-1.

10 ml of Affi Gel 10 resin (Bio-Rad Laboratories) were washed three times with 50 ml sodium acetate buffer, pH 4,5.

10 ml of monoclonal antibody Helix-1, at a concentration of 5 mg/ml in 0,1 M MOPS, pH 7,5 were added to the washed resin.

After 2 hours incubation at room temperature the resin was washed twice with 50 ml PBS.

Possible residual active groups were then saturated by incubating the resin for 2 hours with 20 ml of 0,1 M ethanolamine, pH 8,5.

Finally the antibody coupled matrix was washed with 10 ml 0,1 M sodium acetate buffer, pH 4,0 and with 100 ml PBS.

Antibody Helix-1 covalently bound to Affi-Gel 10 resin was used in affinity cromatography for the antigen purification.

An antigenic preparation constituted by a sonicated suspension of Helicobacter pylori (20 ml) was loaded on the the cromatographic column at a rate flow of 1ml/min by means of a peristaltic pump.

After having washed the resin with 100 ml PBS, the antigen bound to Helix-1 antibody was removed from the column by using 20 ml of 10 mM HCl.

The antigenic acid solution obtained by the column was immediately neutralized with 2 ml of 1 M Na_2HPO_4.

The molecular weight of the *Helicobacter pylori* molecule purified by using antibody Helix-1 was determined through electrophoresis in polyacrylamide gel (SDS-PAGE) according to the technique disclosed by Laemmli in 1970 (Nature, 227, 680-

685).

The affinity purified Helicobacter pylori molecule was diluted 1:2 with SDS-PAGE sample buffer (120 mM Tris, pH 6,8; glycerol 10%, SDS 2%, 2-mercaptoethanole 5%, bromophenol blue 0,002%, and mantained for 5 minutes at 100°C.

20 ml solution were loaded on a 15% polyacrilamide gel.

The electrophoresis was carried out at 40 mA (constant current) for 2 hours at room temperature.

The molecular weight of *Helicobacter pylori* antigen, as purified by means of affinity cromatography, was determined by using a mixture of standard molecular weights (from 14,4 kDa to 97 kDa - Bio-Rad Laboratories).

The gel was stained with the Silver-stain technique by using the Bio-Rad kit.

The electrophoretical analysis of the antigenic preparation obtained through affinity cromatography allowed the identification a single electrophoretical band which, when compared to the standard molecular weights, showed an apparent molecular weight of 16 ± 2 kDa relative to the used standard molecular weights.

Size exclusion liquid cromatography was used in order to gauge the molecular weight of the antigen recognized by the Helix-1 monoclonal antibody, the used condition allowing to keep the molecule in a native form.

The cromatographic process was carried out by using a movable phase constituted by a buffer phosphate, pH 7,4 (PBS Dulbecco).

The molecular weight was calculated by previously gauging the cromatography column with a set of standard molecular weights of Bio-Rad.

The molecular weight, calculated in native conditions by means of size exclusion liquid cromatography, was comprised

between 400 and 600 kDa.

DESCRIPTION OF SOME PREFERRED FORMS OF EMBODIMENT

As previously mentioned, the invention refers to all the analysis methods, both of the immunologic and the chemical-physical type, in order to determine the presence of a Helicobacter pylori molecule having an apparent molecular weight of 16 ± 2 kDa in a solid and/or liquid sample.

In this context, we note that the Helix-1 monoclonal antibody produced by hybridoma 2H11 specifically recognizes a molecule having an apparent molecular weight of 16 ± 2 kDa produced by *Helicobacter pylori*.

According to an important feature of the present invention, Helix-1 antibody represent a tool for uniquely identifying a molecule having an apparent molecular weight of 16 ± 2 kDa produced by *Helicobacter pylori*.

Therefore, the presence of this molecule, in a solid or in a liquid sample, may be determined either by chemical-physical tests (i.e. through cromatography) or by immunological tests by using monoclonal and/or polyclonal antibodies or immunologically active fragments of the latter or by means of affinity reactions with other molecules (e.g. cell receptors, lectines, sugars, phospholipides, enzymes, peptides, etc.).

While carrying out an assay method in order to ascertain the presence, in any kind of sample, of a molecule having an apparent molecular weight of 16 ± 2 kDa produced by *Helicobacter pylori*, according to the present invention it is particularly advantageous to use monoclonal antibody Helix-1.

According to a particularly advantageous form of embodiment of the invention, a method for ascertaining the presence of the antigen having an apparent molecular weight of

16 ± 2 kDa produced by *Helicobacter pylori* comprises the following steps:

a) providing a generic sample to be assayed. This sample is typically provided either as a given quantity of a solid means which is suitably treated before the assay, or as a suspension obtained by a solid sample, or as a liquid sample;

b) providing a monoclonal and/or polyclonal antibody in a biologically active format suitable for immunoreacting with the antigen having an apparent molecular weight of 16 ± 2 kDa produced by *Helicobacter pylori*;

c) admixing the sample with the antibody of step b) in order to form an immunoreaction admixture;

d) keeping said admixture in suitable conditions for enhancing the reaction between antigen and antibody for a period of time ranging from some minutes to some hours;

e) ascertaining whether an antigen-antibody reaction has occurred, and then determining whether the sample shows the molecule having an apparent molecular weight of 16 ± 2 kDa produced by *Helicobacter pylori* and, when possible, determining its concentration.

The analytical immunological method described above may be carried out by using a plurality of methods and realisation formats which are well known to the immunodiagnostics skilled men.

In the prototypes in which the ascertainment of the presence of the antigen having an apparent molecular weight of 16 ± 2 kDa produced by *Helicobacter pylori* takes place by means of a labeled antibody, e.g. by means of a radioactive chemiluminescent isotope, an enzyme or a fluorescent substance, said detecting antibody may be constituted by the

monoclonal and/or polyclonal antibody in its full or fragmented format, provided that it reacts with a molecule having an apparent molecular weight of 16 ± 2 kDa produced by Helicobacter pylori.

Although the following immunologic assay methods use solid phase formats (heterogeneous immunological assay methods), the invention is not limited to the latter, and it includes the homogeneous immunological assay methods too.

A solid matrix affixed to a biologic molecule is conventionally defined as "solid phase".

The heterogeneous immunological assay methods may be prepared by affixing a solid matrix to the Helix-1 monoclonal antibody as well as to other monoclonal and/or polyclonal antibodies directed against a molecule having an apparent molecular weight of 16 ± 2 kDa of *Helicobacter pylori* and obtained by animals (mice, rats, rabbits, chickens, sheep etc.) immunized with *Helicobacter pylori* and/or a molecule having an apparent molecular weight of 16 ± 2 kDa of *Helicobacter pylori*.

Another kind of solid phase may be obtained by binding to a solid matrix the antigen having an apparent molecular weight of 16 ± 2 kDa of *Helicobacter pylori* or *Helicobacter pylori* preparations containing the antigen of 16 ± 2 kDa of *Helicobacter pylori*.

A solid matrix used in heterogeneous immunoassays includes dextran, agarose, polystirene beads, PVC, polyacrilamide, nitrocellulose od nylon-based webs such as sheets, strips or paddles or tubes, plates or the wells of a microtiter plate.

Furthermore, other solid matrices constituted by sinthetic polymers suitable for performing agglutination tests are

constituted by polystirene, divynilbenzene, ethylendimetacrylate, etc.

The presence of an antigen having an apparent molecular weight of 16 ± 2 kDa of *Helicobacter pylori* in solid or liquid samples can be assayed both by competitive or noncompetitive immunoassay methods.

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EXAMPLE 1

Latex agglutination test to detect the antigen having the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori*.

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Covalent binding of the monoclonal antibody Helix-1 to carboxylated latex

The purified monoclonal antibody Helix-1 was covalently coupled to a carboxylated latex (Bang's Laboratories) using as binding chemical reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and N-hydroxysuccinimide (NHS).

1 ml of carboxylated latex beads (1 µm diameter) at 10% of solids is diluted ten times with deionized water and centrifuged at 6.000 revolutions for 30 minutes.

After having sucked the supernatant, the pellet was washed three times with 30 ml of deionized water by centrifugation.

After the last washing step the pellet was resuspended with 5 ml of deionized water.

5 ml of a mixture composed of EDAC/NHS (200 mM/50 mM in water solution) was added to the latex suspension.

After 2 hours of incubation at room temperature the latex particles were washed three times with 10 ml of deionized water.

After the last washing step 5 ml of Helix-1 solution (0,4 mg/ml in 20 mM Hepes buffer; pH 7,3) was added to the pellet.

Thus the suspension was incubated for 18 hours at 4°C.

Finally, the latex suspension was washed twice with deionized water and, to deactivate eventual active groups, 10 ml of 0,1 M ethanolamine, pH 8,3 was added.

After two hours of incubation, three washing steps with 30 ml PBS were carried out.

The pellet obtained after the last washing step was diluted so as to obtain a solid quantity of 0,5% using a buffer constituted by 10 mM Hepes pH 7,4 and containing 10 mg/ml BSA Fraction V, 1mM EDTA, 0,05% Brij and 0,045% sodium azide, 100 µg/ml of mouse non-specific immunoglobulins.

Agglutination reaction of latex particles activated by Helix-1 by means of adding antigen with the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori*.

The latex agglutination test was carried out using the latex microparticles to which the Helix-1 antibodies have been covalently bound.

Providing said reagent as a kit allows to determine the presence of the *Helicobacter pylori* and/or the antigen having the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori* according to the following steps.

The suspension of microparticles coated with Helix-1 (30 µl) is dispensed on a glass slide for agglutination assays.

The sample to be examined, e.g. bacteric colonies or a bioptic fragment drawn during endoscopic examination, is added to the suspension of microparticles conjugated to Helix-1 antibody.

The mixture constituted by sample and microparticles is

mixed with a sterile stick for about 10 - 20 seconds.

The latex suspension containing the sample to be assayed is gently mixed for other 30 seconds moving the glass slide.

During the 30 seconds of incubation the latex particles activated by Helix-1 react with the antigen of the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori* producing a visible mass or agglutant.

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EXAMPLE 2

IMMUNOENZYMATIC METHODS (ELISA) FOR THE SEARCH OF THE ANTIGEN HAVING APPARENT MOLECULAR WEIGHT OF 16 ± 2 kDa OF THE *HELICOBACTER PYLORI*.

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Non-competitive heterogeneous ELISA

Preparation of the solid phase.

Purified Helix-1 immunoglobulins or monoclonal and/or polyclonal antibodies recognizing the antigen of the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori* are absorbed on the plastic material of the microtiter plate wells (NUNC, Maxisorp) by adding 100 µl of a solution of antibodies at a concentration of 10 µg/ml in 50 mM sodium carbonate buffer pH 9,6.

The microtiter plate is maintained for 18 hours at room temperature and then washed twice with PBS containing 0,05% Tween 20 (250 µl/well).

Thus the plastic material is saturated by admixing 200 µl of a solution at 2% of bovine albumin, Fraction V, in PBS.

After a further incubation of 18 hours at room temperature the plate is washed three times as described above and dried at 37°C for 30 minutes.

Conjugation of horseradish peroxidase enzyme to Helix-1 monoclonal antibody.

Helix-1 monoclonal antibody purified by ascitic liquid is conjugated to horseradish peroxidase enzyme (HPR) using the method described by Nakane (Nakane, P.K. and Kawaoi, A. 1974. Peroxidase-labeled antibody. A new method of conjugation, J. Histochem. Cytochem., 22, 1084).

The used procedure is as follows: 4 mg of HRP (Sigma HRP type VI) is solubilized in 1 ml of distilled water, then 0,1 ml of 0,1 M sodium metaperiodate in water is added to HRP solution.

After incubation of 20 minutes in the dark, the HRP solution is dialyzed against 10 mM sodium acetate buffer at pH 4,5.

1 mg of purified monoclonal antibody (1 mg/ml in 0,1M sodium bicarbonate) is added to HRP solution and the obtained mixture is incubated for 2 hours in the dark.

Then, to HRP-antibody mixture 100 µl of a sodium borohydride solution (4 mg/ml in water) is successively added.

After 2 hours of incubation at 4°C, the antibody conjugated to HRP is purified from the mixture by precipitation adding an equal volume of ammonium sulphate saturated solution.

After a washing phase, the precipitate is resuspended with 1 ml of PBS and 1 ml of glycerine and maintained at -20°C.

Non-competitive heterogeneous ELISA for the search of the antigen.

50 µl of samples, positive and negative controls, is admixed in duplicate in the wells of the microtiter plate previously prepared.

In all the wells is added 50µl of Helix-1 conjugated to HRP. After 10 minutes of incubation the wells are washed three

times with PBS containing 0,05% Tween 20.

100 µl of chromogen solution containing the substrate for HRP enzyme (0,1M sodium citrate buffer, pH 4,7 containing 1 mg/ml of urea peroxide and 1 mg/ml of o-phenylendiamine) is added to each well of the microtiter plate.

Then the enzymatic reaction is stopped by adding 50 µl of HCl 2N to each well.

The optical density of the coloured solutions present in the wells is determined at 490 nm using a microtiter plate reader.

In said non-competitive test ELISA, the higher is the colour intensity present in the microtiter plate wells, the higher is the quantity of the antigen having the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori* in the assayed sample.

Competitive heterogeneous ELISA for the search of the antigen.

The antigen having the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori* or the crude sonicate of *Helicobacter pylori* is affixed to the microtiter plate wells (Nunc, Maxisorp) by admixing 0,2 ml of PBS containing 2 µg/ml of antigen or of sonicate cells.

After an incubation of 18 hours at room temperature, the wells are washed three times with 250 µl of PBS containing 0,05% Tween 20.

The plastic material is saturated as described above for the non-competitive ELISA test.

50 µl of positive controls, negative controls and samples, and 50 µl of Helix-1 conjugated to HRP is admixed in duplicate into each well of the microtiter plate.

The so formed mixture is maintained for 30 minutes at room

temperature.

The microtiter plate wells are washed three times as described above.

The quantity which is bound to the solid phase of Helix-1 antibody conjugated to HRP is determined as described in the non-competitive test.

In said immunoenzymatic competitive test, the higher is the colour intensity present in the microtiter plate wells after the enzymatic reaction, the lower is the quantity of the antigen having the apparent molecular weight of 16 ± 2kDa of the *Helicobacter pylori* in the analyzed sample.

CLAIMS

1. A hybridoma (2H11) which produces a monoclonal antibody (Helix-1) that immunoreacts with an antigen of *Helicobacter pylori* having an apparent molecular weight of 16 ± 2kDa, said hybridoma being identified by deposit number ECACC n. 96122033.

2. A monoclonal antibody (Helix-1) which specifically immunoreacts with an antigen of *Helicobacter pylori* that has an apparent molecular weight of 16 ± 2kDa, and is secreted by the hybridoma (2H11) according to claim 1.

3. A molecule having an apparent molecular weight of 16 ± 2 kDa of *Helicobacter pylori* present in any kind of sample, solid or liquid, as indicator of the presence of the bacterium *Helicobacter pylori* in the place from which said sample derives.

4. Molecule according to claim 3, in which said place is a human or animal organism, or the environment, e.g. an aliment, or a culture in vitro for a diagnostic purpose.

5. Molecule according to one of claims 3 or 4, characterized in that its molecular weight, estimated by means of size molecular exclusion chromatography, in non-denaturing conditions, is comprised from 400 to 600kDa.

6. Molecule according to anyone of claims from 3 to 5, characterized in that it is specifically and univocally identified, by means of immunoreaction, by a monoclonal antibody (Helix-1) secreted by a hybridoma (2H11) identified by the deposit number ECACC n. 96122033.

7. Method to detect the presence of a molecule according to anyone of claims from 3 to 6, in a sample both solid and liquid, characterized in that it is carried out by means of chemical-physical tests, e.g. chromatography, or immunological tests using monoclonal and/or polyclonal

antibodies or immunological active fragments thereof or by means of affinity reactions with other molecules, e.g. cellular receptors, lectines, sugars, phospholipides, enzymes, peptides, etc..

8. Method according to claim 7, characterized in that it comprises the following steps:

a) providing for a generic sample to be assayed said sample being typically provided either as a predetermined quantity of a solid medium which is properly treated before being assayed, or as a suspension obtained by a liquid sample, or as a liquid sample;

b) providing for a monoclonal and/or polyclonal antibody in a biological active state which immunoreacts with the antigen having an apparent molecular weight of 16 ± 2kDa of *Helicobacter pylori*;

c) admixing the sample with the antibody of phase b) so as to obtain an immunoreaction mixture;

e) keeping the mixture under suitable conditions so as to enhance the reaction between the antigen and the antibody for a period of time ranging from few minutes to hours;

f) ascertaining whether an antigen/antibody reaction has occured and then determining the presence, in the assayed sample, of the molecule having an apparent molecular weight of 16 ± 2kDa of *Helicobacter pylori*, and determining its concentration.

9. Method according to claim 7, characterized in that the presence of antigen of the apparent molecular weight of 16 ± 2kDa of *Helicobacter pylori* in both liquid and solid samples is ascertained by means of immunological dosage methods of competitive and non-competitive type.

10. Method according to claim 7, characterized in that the presence of antigen of the apparent molecular weight of 16 ± 2kDa of *Helicobacter pylori* in both liquid and solid samples is ascertained by means of immunological heterogeneous or omogeneous dosage methods.

11. Use of the specific immulogical recognition of a monoclonal antibody (Helix-1), or of fragments thereof or of monoclonal and/or polyclonal antibodies or of fragments thereof capable of specifically recognize by means of immunoreaction an antigen or molecule of apparent molecular weight of 16 ± 2kDa in order to detect in a sample the presence of *Helicobacter pylori*, or of the antigen of 16 ± 2kDa or of antigen aggregates of 16 ± 2kDa, or of antigen fragments of 16 ± 2kDa.

12. Use according to claim 11, characterized in that said monoclonal antibody (Helix-1) is secreted by the hybridoma (2H11) identified by the deposit number ECACC n. 96122033.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To
Sanitaria Scalingera SPA
Via Della Consortia
2 ZAI-37127 Avesa (Verona)
Italy

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
2H11	96122033

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 2/12/96 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Dr A Doyle

Address: ECACC, CAMR
Porton Down
Salisbury, SP4 OJG

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 22.4.97

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
Sanitaria Scaligera SPA
Via Della Consortia
2 ZAI-37127 Avesa (Verona)
Italy

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I. DEPOSITOR		II. IDENTIFICATION OF THE MICROORGANISM
Name:	Sanitaria Scaligera SPA	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: 96122033
Address:	Via Della Consortia 2 ZAI-37127 Avesa (Verona) Italy	Date of the deposit or of the transfer: 2/12/96

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2/12/96 [2]. On that date, the said microorganism was

[3] ☐ viable

[3] ☐ no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Dr A Doyle Address: ECACC, CAMR Porton Down Salisbury, SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 22.4.97

[4] Fill in if the information has been requested and if the results of the test were negative.

Form BP/9 (second and last page)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/00299

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/20 C07K16/12 C07K14/205 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 33220 A (CSL LTD. & THE UNIVERSITY OF NEW SOUTH WALES) 24 October 1996 see examples see claims ---	3,4,7-10
X	WO 96 01272 A (RICAN LTD.) 18 January 1996 see claims ---	3,4,7-10
A	U. RODEWIG ET AL.: "Evaluation of a monoclonal antibody for detection of Helicobacter pylori in a direct immunofluorescence test." EUROPEAN JOURNAL OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES, vol. 11, no. 8, August 1992, BRAUNSCHWEIG, GERMANY, pages 737-739, XP002063605 see the whole document --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 1998	20/05/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

[redacted] ional Application No
[redacted]/IT 97/00299

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Y. SHOWJI ET AL.: "Seroprevalence of Helicobacter pylori infection in patients with connective tissue diseases." MICROBIOLOGY AND IMMUNOLOGY, vol. 40, no. 7, 1996, TOKYO, JAPAN, pages 499-503, XP002063606 see abstract see table 1 see page 502, left-hand column, line 9 - line 16 ---	1-12
A	CHEMICAL ABSTRACTS, vol. 118, no. 15, 12 April 1993 Columbus, Ohio, US; abstract no. 145558c, K-H. RHEE ET AL.: "Production of the monoclonal antibody and the genomic library of Helicobacter pylori." page 652; column 1; XP002063607 see abstract & TAEHAN MISAENGMUL HAKHOECHI, vol. 26, no. 4, 1991, KOREA, pages 305-316, ---	1-12
P,X	WO 97 03360 A (ORAVAX, INC.) 30 January 1997 see the whole document -----	1-12

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible]l Application No
PCT/[illegible] 97/00299

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9633220 A	24-10-96	AU 5262196 A	07-11-96
		CA 2217496 A	24-10-96
		EP 0821698 A	04-02-98
WO 9601272 A	18-01-96	AU 2751395 A	25-01-96
		AU 2751495 A	25-01-96
		CA 2194236 A	18-01-96
		CA 2194237 A	18-01-96
		EP 0769018 A	23-04-97
		EP 0769019 A	23-04-97
		GB 2303854 A	05-03-97
		GB 2303855 A	05-03-97
		IE 950496 A	10-01-96
		IE 950497 A	10-01-96
		WO 9601273 A	18-01-96
		JP 10502365 T	03-03-98
		JP 10502366 T	03-03-98
		ZA 9505499 A	02-01-96
		ZA 9505500 A	02-01-96
WO 9703360 A	30-01-97	AU 6345396 A	10-02-97